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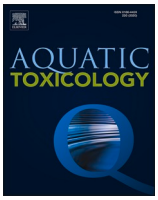

Scoping intergenerational effects of nanoplastic on the lipid reserves of Antarctic krill embryos

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ABSTRACT

Antarctic krill (*Euphausia superba*) plays a central role in the Antarctic marine food web and biogeochemical cycles and has been identified as a species that is potentially vulnerable to plastic pollution. While plastic pollution has been acknowledged as a potential threat to Southern Ocean marine ecosystems, the effect of nanoplastics (<1000 nm) is poorly understood. Deleterious impacts of nanoplastic are predicted to be higher than that of larger plastics, due to their small size which enables their permeation of cell membranes and potentially provokes toxicity. Here, we investigated the intergenerational impact of exposing Antarctic krill to nanoplastics. We focused on whether embryonic energy resources were affected when gravid female krill were exposed to nanoplastic by determining lipid and fatty acid compositions of embryos produced in incubation. Embryos were collected from females who had spawned under three different exposure treatments (control, nanoplastic, nanoplastic + algae). Embryos collected from each maternal treatment were incubated for a further 6 days under three nanoplastic exposure treatments (control, low concentration nanoplastic, and high concentration nanoplastic). Nanoplastic additions to seawater did not impact lipid metabolism (total lipid or fatty acid composition) across the maternal or direct embryo treatments, and no interactive effects were observed. The provision of a food source during maternal exposure to nanoplastic had a positive effect on key fatty acids identified as important during embryogenesis, including higher total polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) when compared to the control and nanoplastic treatments. Whilst the short exposure time was ample for lipids from maternally digested algae to be incorporated into embryos, we discuss why the nanoplastic-fatty acid relationship may be more complex. Our study is the first to scope intergeneration effects of nanoplastic on Antarctic krill lipid and fatty acid reserves. From this, we suggest directions for future research including long term exposures, multi-stressor scenarios and exploring other critical energy reserves such as proteins.

1. Introduction

Over 8300 million metric tonnes (Mt) of virgin plastics have been generated since plastic first became commonly used in the 1950's (Geyer et al., 2017) with an estimated 4.8 – 12.7 Mt entering the global ocean in 2010 alone (Jambeck et al., 2015). As such, oceanic plastics are now considered to be verging on a planetary boundary threat (Arp et al., 2021) with concentrations of plastic contaminants in the natural environment exceeding thresholds anticipated to lead to global effects in vital earth-system processes (Villarrubia-Gómez et al., 2018). The Southern Ocean, surrounding Antarctica, whilst being one of the most isolated regions of the world, is not devoid of plastic pollution. Plastic from the macro to micro scale has been detected throughout the Antarctic marine ecosystem, from surface waters (e.g., Jones-Williams et al., 2020; Suaria et al., 2020) to the seafloor (e.g., Munari et al., 2017; Reed et al., 2018). Nanoplastic (<1000 nm) (Hartmann et al., 2019) derives from larger plastics breaking down due to ultraviolet (UV) radiation and mechanical weathering, biodegradation, or digestive fragmentation, as

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well as from primary sources such as 3D printing, biomedical products, and drug delivery (Alimi et al., 2017; Galloway et al., 2017). The distribution and abundance of nanoplastics within the natural environment is yet to be determined, due to the difficulty of environmental sampling (Lv et al., 2020). However, a high proportion of plastic in the Southern Ocean is anticipated to have travelled long distances from source regions north of the Southern Ocean (Obbard, 2018). Long-term exposure to elevated UV due to a seasonally thinned stratospheric ozone over Antarctica, and rough seas means plastics may be highly susceptible to fragmentation in the Southern Ocean (Rowlands et al., 2021 a).

Whilst the biological impact of microplastics (1-1000 μm) (Hartmann et al., 2019) on marine organisms has received widespread attention, with deleterious impacts on zooplankton found to include inhibited energy reserves, growth and survival, and disrupted behaviour and reproduction (e.g. Cole et al., 2013; Coppock et al., 2019; Wright et al., 2013), the effects of nanoplastics are less well studied (Koelmans, 2019). For marine zooplankton, nanoplastic exposure has been associated with negative impacts ranging from increased oxidative stress to lethality (Corsi et al., 2020). Nanoplastics can pose heightened risks compared to larger plastic particles owing to their increased ability to translocate and permeate cell membranes, which makes cellular uptake routes possible. For example, nanoplastics have been shown to translocate into the digestive gland of sea urchin embryos (della Torre et al., 2014), to the tissues and haemolymph of mature bivalves (Sendra et al., 2020) and to become internalised by immune cells of adult sea urchins (Majeske et al., 2013). Nanoplastics may also have a longer retention time inside the body compared to microplastics (Jeong et al., 2016) and studies are beginning to consider the potential long-term impacts of nanoplastic, such as transfer to embryos. Intergenerational transfer of nanoplastic has been observed in zebrafish (Pitt et al., 2018) and temperate soil nematodes Zhao et al., (2017). For medaka (*Oryzias melastigma*), a species often found in fresh/brackish water in east and south Asia, parental exposure to nanoplastic led to the detection of nanoparticles in embryos, decreasing the bodyweight of first-generation males as well as changing the composition of their gut microbiota. Furthermore, even without maternal transfer of particles, offspring of parentally-exposed marine medaka displayed a significantly reduced hatch success, heart rate and body length (Wang et al., 2019).

Antarctic krill (Euphausia superba), hereafter krill, have a critical role in the Southern Ocean ecosystem, transferring energy from primary producers to higher predators (Atkinson et al., 2012), playing a key role in biogeochemical cycles (Belcher et al., 2019; Manno et al., 2020) and having great economic importance as the most fished species by tonnage in the Southern Ocean (Nicol et al., 2012). Krill have been identified as potentially vulnerable to plastic pollution owing to their indiscriminate feeding habits, aligned with their high grazing rates and strong association with sea ice (a known sink for plastic particulates) (Dawson et al., 2018; Kelly et al., 2020; Rowlands et al., 2021b a). Further, krill has limited genetic variability and known vulnerabilities to other anthropogenic stressors, for example ocean warming and ocean acidification (Rowlands et al., 2021 a). Bergami et al., (2020) demonstrated that nanoplastic exposure impacts juvenile krill by reducing swimming activity and increasing the frequency of exuviae (exoskeleton) production. Nanoplastic (160 nm) exposure combined with ocean acidification has also been demonstrated to hinder the development of directly exposed krill embryos, reducing the likelihood of them reaching later development stages by approximately 9% (Rowlands et al., 2021 b).

To assess the potential energetic costs for krill embryos of exposure to nanoplastics in this study, we analysed lipid accumulation and lipid/fatty acid composition, a key energy resource for krill embryonic development (Amsler and George, 1985; Yoshida et al., 2011). Lipid droplets contain organelles that synthesise vitellogenin, the egg-yolk precursor granules (Sun and Zhang, 2015). Yolk granules may also be a potential maternal route for nanoplastic transfer (Brun et al., 2017) since many plastics have lipophilic properties which can cause them to accumulate in or on lipid droplets (Rosenkranz et al., 2009). Nanoplastic exposure has already been noted to impact lipid levels of other species, for example, Cui et al., (2017) reported heightened levels of lipid storage in Daphnia galeata embryos following exposure to nanoplastic particles in the brood chamber, which they deemed to be a stress response to the embryos experiencing abnormal conditions. Cedervall et al., (2012) also determined that polystyrene nanoparticles are transferred through the marine food web and negatively impact lipid metabolism of the top consumer via nanoplastic binding to apolipoprotein A-I, a fundamental component of the fat metabolism in most organisms.

In this study, we investigated whether polystyrene nanoplastic particles (50 nm) can have an intergenerational effect on krill. We investigated responses of control and maternally exposed krill embryos to nanoplastic at varying concentrations, assessing their total lipid mass and lipid and fatty acid profiles. We explored responses with and without the addition of a natural food source, since the toxicity of plastic particles to marine species has been observed to differ based on food availability (Piccardo et al., 2020). Results enhance our understanding of krill's energetic responses to nanoplastic at one of their potentially most sensitive life-stages.

2. Methods

2.1. Nanoplastic preparation and characterisation

Fluorescent 50 nm diameter amino modified polystyrene (PS-NH2) spheres (2.5% w/v, 5 ml, Magsphere) were purchased without stabilisers/bacteriostatic preservatives such as sodium azide (NaN3), given the potential for such additives to have toxic effects on organisms (Bergami et al., 2017; Manfra et al., 2017). Positively charged (aminated) particles were selected to align with the previous research on krill (Bergami et al., 2020), because aminated particles are generally better dispersed in saline conditions (della Torre et al., 2014), and because they have been shown to have increased toxicity to marine organisms compared to negatively charged particles in other nanoplastic incubation experiments (della Torre et al., 2014).

To understand the behaviour (surface charge and aggregation state) of polystyrene nanoplastic during incubation experiments, we tested the stability of particles incubated in 0.22 μm filtered seawater (FSW) and 0.22 μm Milli-Q water. Seawater was collected from Cumberland Bay, South Georgia (54.260°S, 36.439°W), close to the krill sampling site. Nanoplastic stock was vortexed for 30 s and sonicated in a bath for 60 s prior to dilution (50 μg mL–1). Dynamic Light Scattering (DLS) analyses (Zetasizer Ultra; Malvern Instruments) determined the aggregation state (polydispersity index, PDI), the hydrodynamic diameter of particles/aggregates (Z-average; nm) and the mean surface charge (ζ-potential; mV) of the nanoplastic. Measurements were performed in triplicate in 50 μg mL–1 nanoplastic suspensions at T0 and T24h both at 2 °C to reflect the maternal stress incubation and at 0.5 °C to reflect the embryos' incubation.

For experimental treatments, nanoplastic suspensions were freshly prepared in FSW (0.22 μm filtered, T = 2.7 °C, salinity 33.9 psu) collected from the ship's underway pump in the same region as the caught krill. All nanoplastic stock solutions were briefly vortexed (10 s) but not sonicated prior to use, according to (Della Torre et al., 2014).

2.2. Krill collection

Krill were collected from aboard the RRS James Clark Ross in December 2019 (cruise JR19001) using a targeted Rectangular Midwater Trawl system, with a mouth area of 8 m2 and mesh size of 5 mm (RMT8). Sampling took place over the northwest continental shelf of South Georgia, in the northern Scotia Sea. The location of the outbound and inboard trawls were 53.747°S, 38.025°W and 53.767°S, 38.025°W respectively). Active and undamaged gravid females (with spermatophores inside the thelycum) were selected and acclimated in buckets of seawater for 22 hours prior to experimental treatments.

2.3. Experimental set-up

The experimental set-up can be separated into two phases. Firstly, gravid females were incubated in one of four treatments including a control, algae only, nanoplastic only, and nanoplastic plus algae treatment, up to the point of spawning (see maternal treatment 2.3.1). Secondly, produced embryos underwent further incubations through a 6-day development phase (see embryo treatment 2.3.2).

2.3.1. Maternal treatment

For the maternal treatment, singular krill were added to 2 L Kilner incubation jars containing 1000 mL of 0.22 μm FSW (n=5 per treatment). All jars were aerated and contained a plastic mesh inner to allow the krill embryos to sink through and prevent cannibalism and were kept in the onboard cold room (2-4 °C). Krill were left to acclimate for 2 hours prior to treatments being added comprising: control (FSW), algae only, nanoplastic (2.5 μg mL−1) and nanoplastic (2.5 μg mL−1) plus algae (Fig. 1).

For the maternal control treatment, ambient seawater was collected from the ship's underway pump (from approximately 7 m depth) in the same region as the caught krill and filtered through a 0.22 μm membrane. The maternal algae treatment followed the same procedure as the control treatment, but with the addition of 10 μl L−1 of 'ISO 1800', a whole-cell concentrate of Isochrysis microalgae, selected based on its use in other krill incubation experiments (Kawaguchi et al., 2010) and evidence of it being a lipid rich flagellate that can be processed rapidly in the krill gut (Pond, 1993). For the maternal nanoplastic treatment, nanoplastic stocks were prepared to a final concentration of 2.5 μg

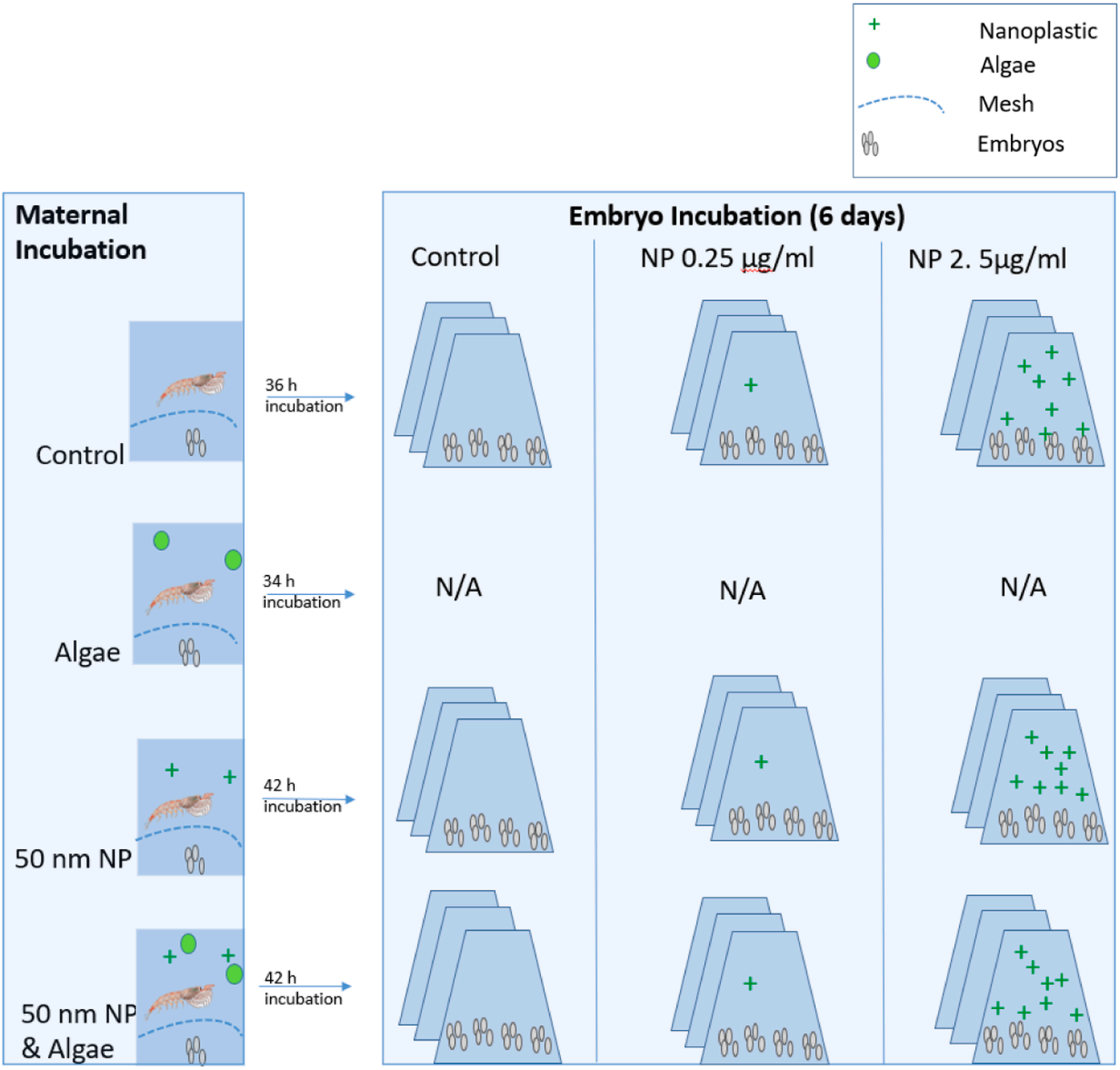

Fig. 1. A schematic of the experimental set-up. Maternal incubation timings 36 h – 42 h were the time taken for the mother to spawn within the treatment water. Maternal incubation contained four treatments: control, nanoplastic (NP), algae, nanoplastic and algae. Produced embryos were collected from one female per treatment and incubated for a further 6 days. Embryo incubation included three treatments: control, nanoplastic 0.25 μg mL^{-1} and nanoplastic 2.5 μg mL^{-1}. From the algae only treatment, there were no fertilised embryos to proceed with the secondary part of the incubation experiment (see 2.3.2 Embryo treatments).

mL−1 in FSW, following previous nanoplastic research on Antarctic krill embryos (Rowlands et al 2021 b) and juveniles (Bergami et al., 2020). The tested concentration corresponded to approximately 3.64 × 1010 nanoplastic particles per mL. This concentration also aligns with observed acute toxicity thresholds for other marine zooplankton that have been incubated with polystyrene nanoplastic (Bergami et al., 2017; Manfra et al., 2017). Finally, the maternal nanoplastic plus algae treatment followed the same procedure as for the nanoplastic treatment, but with the addition of Isochrysis spp as per the algae only treatment.

Females were monitored every four to 6 hours for embryo production. Once a gravid female spawned, embryos were examined under a light microscope to check suitability for further incubation experiments, i.e., successful fertilisation (displayed a layer of fertilisation jelly surrounding the embryo membrane) and normality in terms of shape. Several females spawned from the control (2), nanoplastic (3) and nanoplastic plus algae (3) treatments. However, only embryo batches that were produced after similar incubation times (34-42 hours) were selected to continue the secondary part of the experiment, to ensure comparable findings. Whilst one female in the algae only maternal treatment spawned at a similar time to the other treatments, eggs were not developing past initial fertilisation upon examination under the light microscope. In this instance the eggs did not undergo the follow-on treatment. All microscope work took place in the cold room to minimise the impact of temperature variance on embryos during examination.

2.3.2. Embryo treatments

Embryos were incubated for 6 days at 0.5 °C, a known stable temperature for krill development (Jia et al., 2014) and in line with our previous incubation experiment (Rowlands et al 2021 b). Treatment solutions were added to glass bottles to a total volume of 250 mL. One mother from each of the control, nanoplastic and nanoplastic plus algae maternal treatments spawned after a time in the incubation deemed to be comparable (34–42 hours). Embryos from these mothers were exposed to three follow-on treatments: control (0.22 μm FSW), nanoplastic low dose (0.25 μg mL−1) or nanoplastic high dose (2.5 μg mL−1), each with three replicates and approximately 60 embryos per replicate.

Filtered (0.22 μm) ambient seawater collected from the ship's underway pump in the same region as the caught krill was used in the control treatment, as for the maternal exposure. Nanoplastic was prepared to a final concentration of 0.25 μg mL−1 for the low dose and 2.5 μg mL−1 for the high dose in FSW. The upper plastic concentration was chosen to reflect that of the maternal exposure. Whilst data regarding environmental concentrations of similar nanoplastic particles in sea water are not available, we chose the low dose to be more reflective of anticipated environmental levels, though this may still be higher than conservative global estimates (< 1 μg L−1) (Zhao et al., 2017; Beiras and Schönemann, 2020).

Each day, the treatment water within incubation jars was agitated with a soft-tip pipette to increase dispersion in the seawater and ensure interaction with krill embryos.

2.4. Experimental endpoint

At the experiment endpoint, following the 6-day incubations, embryos were collected from individual glass bottles using a soft-tip pipette and transferred into 3 mL microcentrifuge tubes containing 4% formaldehyde as a preservative.

To prepare the samples for lipid analyses, intact, randomly selected embryos were bathed in Milli-q water (3 × 5 minutes) to remove preservatives and filtered onto 25 mm 0.7 μm glass fibre filters. Sample size ranged between 15-30 embryos per replicate based on the number of suitable (intact) embryos of those allocated for lipids analyses. Filters were placed in open petri dishes, wrapped in pierced foil to allow air exchange, and freeze dried (-60 °C; 10−2 mBar) for 24 hours after two hours storage at -20 °C.

2.5. Lipids & fatty acids

Following the methods of Kiriakoulakis et al., (2004), lipid extractions were carried out on each homogenised, freeze-dried sample (dry weight 0.15 – 0.62 mg per replicate). An internal standard (50 μL of 5α (H)-cholestane; 101 ng μL−1) was added to each, followed by a mixture of dichloromethane (DCM) and methanol (9:1; 15 mL). The lipid extractions were then sonicated twice for 15 min and the resulting extract was decanted into round bottom flasks. The solvent was evaporated to dryness under vacuum using a rotary evaporator at approximately 30 °C. Each sample was then passed through a Pasteur pipette filled with anhydrous sodium sulphate using DCM (3 mL). The solvent was blown down with nitrogen gas and the samples were stored (-20 °C) before transmethylation (10% acetyl chloride/methanol; Christie, 1982), passed through a Pasteur pipette filled with anhydrous potassium carbonate and blown down under nitrogen gas to dryness. The samples were finally derivatised with N,O Bis(trimethylsilyl)trifluoroacetamide (BSTFA; 50 μL, 40 °C, 45 min), blown down under nitrogen gas, and stored (-20 °C) until analysis.

Gas chromatography mass spectrometry (GC-MS) analyses were conducted using a GC Trace 1300 fitted with a split-splitless injector and column DB-5MS (60 m x 0.25 mm (internal diameter), with film thickness 0.1 μm, non-polar stationary phase of 5% phenyl and 95% methyl silicone), using helium as a carrier gas (2 mL min−1). The GC oven was programmed after 1 minute from 60 °C to 170 °C at 6 °C min−1, then from 170 °C to 315 °C at 2.5 °C min−1 and held at 315 °C for 15 min. The eluent from the GC was transferred directly via a transfer line (320 °C) to the electron impact source of a Thermoquest ISQMS single quadrupole mass spectrometer. Operating conditions were ionisation potential 70 eV; source temperature 215 °C; trap current 300 μA. Mass data were collected at a resolution of 600, cycling every second from 50–600 Daltons and were processed using Xcalibur software.

Compounds were identified either by comparison of their mass spectra and relative retention indices with those available in the literature and/or by comparison with authentic standards (fatty acids, alcohols and sterols). Quantitative data were calculated by comparison of peak areas of the internal standard with those of the compounds of interest, using the total ion current (TIC) chromatogram. The relative response factors of the analytes were determined individually for 36 representative fatty acids, sterols and an alkenone using authentic standards. Response factors for analytes where standards were unavailable were assumed to be identical to those of available compounds of the same class.

Targeted fatty acids were among those identified to be associated with krill embryogenesis by Yoshida et al (2011) including 5,8,11,14, 17-eicosapentaenoic acid (EPA) and 4.7.10,13,15,19-docosahexaenoic acid (DHA), which are critical in brain and neurological development (Persson and Vrede, 2006), as well as the EPA/DHA ratio. Linoleic acid (9,12-octadecenoic acid) variations were also explored because of its importance during embryogenesis (Yoshida et al., 2011). Dietary markers 9-hexadecenoic acid (16:1 (n-7)) and hexadecenoic acid (16:0) were also assessed.

2.6. Nanoplastic internalisation

To investigate whether nanoplastic had been internalised by incubated krill embryos, we assessed a subsample of embryos using Scanning Electron Microscopy (SEM, Zeiss GeminiSEM 500). Embryos from two conditions were used for analyses (a maternal control / embryo nanoplastic high dose (2.5 μg mL−1) treatment and a maternal nanoplastic (2.5 μg mL−1) / embryo nanoplastic high dose (2.5 μg mL−1) treatment (n = 10 per treatment, split between replicates). Embryos were first cleansed of the fixative with Milli-Q water (3 × 5 minute bath) and next an ethanol gradient before being digested (10% KOH, 60 °C) and filter

papers (Whatman Anodisk, diameter 25mm, poresize 20 nm) were assessed via SEM after application of a 5 nm gold/palladium coating.

2.7. Data analyses

Differences between samples (the homogenate of embryos, n=3 per treatment combination) were tested using ANOVA. Two-way ANOVA and Tukey's post hoc comparisons were performed to compare the means of total lipid and fatty acid composition after 6 days of development in each experimental treatment. Assumptions of normality were assessed by visual inspection of Normal Q-Q plots for all combinations of maternal treatment (control, nanoplastic, nanoplastic plus algae) and embryo treatment (control, nanoplastic low dose, nanoplastic high dose) or Shapiro-Wilk's test ($p>0.05$). Statistical analyses were conducted using SPSS 28.0.1.0.

3. Results

3.1. Behaviour of polystyrene nanoplastic in antarctic seawater

Characterisation data from DLS analysis are reported as mean ± Standard Deviation (SD, n = 3). DLS analyses revealed a consistent dispersion and stability of nanoplastic in Milli-Q over 24 h at 0.5 °C and 2 °C as indicated by the minimal change (<20 nm) in Z-average resembling manufacturer specifications and low PDI values (Table 1). In contrast, agglomerates of nanoplastic particles were observed in seawater, with measurements at the maternal incubation temperature surpassing current upper thresholds for particles exhibiting nano-specific behaviours (1000 nm) after 24 h (Z-average, 2156.66 ± 700.84 nm).

3.2. Nanoplastic internalisation

Through qualitative analyses, nanoplastic particles were identified in the digested residual of embryos from both tested treatments (maternal control / embryo nanoplastic, and maternal nanoplastic / embryo nanoplastic conditions) (Fig. 3).

3.3. Lipids and fatty acids

Average total lipid concentrations per treatment ranged between 106.87 – 161.32 mg g−1 (Table 2). The major fatty acids of the krill embryonic stage were derived from their triacylglycerols 18:1 (n-9c), 14:0, 16:0 accounting for 60.4 – 79.7% of the total fatty acids across individual samples whilst EPA and DHAs only occurred in small amounts 1.3 – 11.3% (Table 3).

The total lipid (mg g−1) was not significantly affected by either maternal treatment ($F= 2.944$, $p = 0.078$) or embryo treatment ($F = 1.064$, $p = 0.106$) and there was no significant interaction effect between the two treatments ($F = 0.798$, $p = 0.542$). However, total polyunsaturated fatty acids (PUFAs) differed significantly based on both maternal treatment ($F = 14.78$, $p = <0.01$) and embryo treatment ($F = 4.120$, $p = 0.034$) (Fig. 2). The nanoplastic plus food maternal treatment showed significantly higher total PUFA than both the control and the nanoplastic treatment ($p = <0.01$) whilst the nanoplastic low dose embryo treatment showed significantly lower total PUFAs than the control ($p = 0.031$) but not the nanoplastic high dose. Total mono-unsaturated fatty acids (MUFAs) also differed significantly based on maternal treatment ($F = 14.590$, $p = <0.001$) with the nanoplastic plus food maternal treatment showing significantly lower total MUFA than both the control and the nanoplastic treatment ($p = <0.01$), there was no difference between embryo treatments ($F = 1.943$, $p = 0.172$). Further, there was no significant interaction effect between the two treatment variables for either total PUFAs ($F = 0.170$ $p = 0.951$) or total MUFAs ($F = 0.902$, $p = 0.484$).

The EPA and DHA significantly differed based on maternal treatment ($F= 52.759$, $p = <0.01$ and $F = 72.712$ $p = <0.001$ respectively) with the nanoplastic plus food treatment being significantly higher than both the control and nanoplastic only treatment in both cases ($p = <0.01$). There was no significant difference between embryo treatments ($F = 1.423$, $p = 0.267$ and $F = 1.503$, $p = 0.249$ respectively), and there was no significant interaction effect ($F = 0.619$, $p = 0.655$ and $F = 0.998$, $p = 0.434$ respectively). There was no significant difference in the EPA/DHA ratio between maternal treatments ($F = 1.289$, $p = 0.300$) or embryo treatments ($F = 0.950$, $p = 0.405$), and no interaction effect between the two ($F= 1.004$, $p = 0.431$). Linoleic acid did not significantly differ based on maternal treatment ($F = 2.590$, $p = 0.103$) or embryo treatment ($F = 1.864$, $p = 0.184$) and no interaction effect was identified ($F = 0.750$, $p = 0.571$). The diatom dietary marker 16:1 (n-7) significantly differed based on the maternal treatment ($F = 83.605$, $p = <0.01$) with the nanoplastic plus algae treatment having a lower mol% ($p = <0.01$) compared to both the control or nanoplastic only treatment. The flagellate dietary marker 16:0 also differed significantly based on the maternal treatment ($F = 6.138$, $p = 0.009$) with the nanoplastic plus algae treatment having a higher mol % ($p = 0.007$) compared to the control. There was no significant effect of embryo treatment for both 16:1 (n-7) and 16:0 respectively ($F = 0.646$, $p = 0.536$, $F = 0.004$, $p = 0.996$) and no interaction effect ($F= 1.682$, $p = 0.198$, $F = 0.969$, $p = 0.448$).

Table 1
Measurements of the mean surface charge (ζ-potential) confirmed a positive charge of the plastic spheres, with a value of 39.84 ± 1.11mV in Milli-Q, and a smaller absolute value of 4.60 ± 0.45 mV observed in seawater.

Medium	Time (h)	Temperature (°C)	*Z*-average (nm)	Polydispersity index (PDI)
mQW	0	0.5	53.38 ± 0.44	0.12 ± 0.03
mQW	24	0.5	53.37 ± 0.24	0.095 ± 0.02
mQW	0	2	53.42 ± 0.74	0.11 ± 0.03
mQW	24	2	58.27 ± 0.57	0.11 ± 0.02
FSW	0	0.5	57.76 ± 0.81	0.14 ± 0.01
FSW	24	0.5	743.63 ± 131.17	0.30 ± 0.03
FSW	0	2	70.11 ± 0.79	0.16 ± 0.00
FSW	24	2	2156.66 ± 700.84	0.396 ± 0.25

Table 2
Total lipid (mean ± Standard Deviation) of embryos at the experiment endpoint

Maternal & embryo treatment	Total lipid (mg g^{-1})
Maternal control / Embryo control	148.09 ± 60.94
Maternal control / Embryo low dose	106.92 ± 11.97
Maternal control / Embryo high dose	160.87 ± 59.70
Maternal NP / Embryo control	111.88 ± 16.34
Maternal NP / Embryo low dose	119.06 ± 17.69
Maternal NP / Embryo high dose	114.65 ± 13.06
Maternal NP & algae / Embryo control	145.70 ± 6.45
Maternal NP & algae / Embryo low dose	145.53 ± 18.69
Maternal NP & algae / Embryo high dose	161.32 ± 21.49

4. Discussion

We aimed to determine whether embryonic energy resources differed when gravid female krill were exposed to nanoplastic by analysing lipid and fatty acid compositions of embryos produced in incubation. Embryos had typical krill composition, mainly comprising of fatty acids 14:0, 16:0 and 18:1(n-9), the first end-products of fatty-acid biosynthesis. Total lipid reserve ranges (106.87 – 161.32 mg g−1) were similar to the observations of Yoshida et al., (2011) of embryos from both field collected and laboratory reared krill (117.0 – 359.1 mg g−1). We found there was no significant difference in total lipid reserve in embryos spawned from the three maternal treatments that produced developing embryos, suggesting no effect of nanoplastics or algae within

Table 3
Major fatty acid composition (mol %) of embryos after 6 days

	Fatty acid									
Maternal & embryo treatment	14:0 *(a)	16:1(n-7c) *(a)	16:0 *(a)	18:2(n-6) LA *(a)	18:1 (n-9c) *(a)	18:1 (n-7c) *(a)	18:0 * (a)	20:1 (n-9c) *(a)	C20:5 (n-3) EPA *(a)	C22:6 (n-3) DHA *(a)
Maternal control / Embryo Control	10.48 ± 1.28	9.63 ± 2.22	25.37 ± 6.29	4.94 ± 1.56	35.27 ± 14.73	0.13 ± 0.05	2.28 ± 0.66	0.55 ± 0.09	1.76 ± 0.79	0.21 ± 0.13
Maternal control / Embryo low dose	12.77 ± 2.10	10.86 ± 0.22	28.20 ± 2.70	5.81 ± 0.24	25.68 ± 1.07	0.13 ± 0.01	2.24 ± 0.23	0.46 ± 0.02	1.90 ± 0.90	0.25 ± 0.20
Maternal control / Embryo high dose	13.19 ± 1.37	11.13 ± 0.58	27.41 ± 1.04	5.28 ± 0.26	25.41 ± 0.69	0.14 ± 0.02	3.15 ± 0.57	0.64 ± 0.05	2.66 ± 1.33	0.15 ± 0.12
Maternal NP / Embryo Control	11.27 ± 2.71	11.63 ± 0.47	28.67 ± 1.93	4.35 ± 3.71	26.33 ± 2.12	0.16 ± 0.02	3.44 ± 1.10	0.75 ± 0.10	1.53 ± 0.43	0.10 ± 0.03
Maternal NP / Embryo low dose	8.67 ± 0.59	10.83 ± 0.45	28.34 ± 0.55	6.62 ± 0.38	26.54 ± 0.73	0.15 ± 0.01	3.53 ± 1.10	0.54 ± 0.14	2.29 ± 0.70	0.21 ± 0.11
Maternal NP / Embryo high dose	8.45 ± 2.70	10.59 ± 0.63	29.13 ± 1.53	6.86 ± 0.58	26.61 ± 1.08	0.13 ± 0.01	3.73 ± 1.10	0.64 ± 0.13	1.79 ± 0.39	0.07 ± 0.08
Maternal NP & algae / Embryo control	6.67 ± 2.85	5.37 ± 1.04	32.76 ± 1.65	4.31 ± 0.22	25.56 ± 0.54	0.27 ± 0.01	8.70 ± 2.32	0.80 ± 0.05	5.95 ± 2.51	1.39 ± 0.78
Maternal NP & algae / Embryo low dose	6.88 ± 1.74	6.24 ± 0.28	30.25 ± 1.45	4.79 ± 0.17	24.00 ± 1.01	0.26 ± 0.01	6.50 ± 1.49	0.71 ± 0.15	7.77 ± 1.04	2.07 ± 0.41
Maternal NP & algae / Embryo high dose	10.15 ± 1.08	6.24 ± 0.68	30.25 ± 0.54	4.70 ± 0.68	24.00 ± 1.79	0.26 ± 0.03	6.50 ± 2.13	0.71 ± 0.69	7.77 ± 0.79	2.07 ± 0.21

Data are mean ± SD. ANOVA comparing maternal treatment and embryo treatment, asterisks show the significant results, letters show corresponding treatment a = maternal treatment, b = embryo treatment, colour variation is to show where a statistically significant difference between groups exists (p=<0.05). Only maternal treatment (a) showed results of statistical significance.

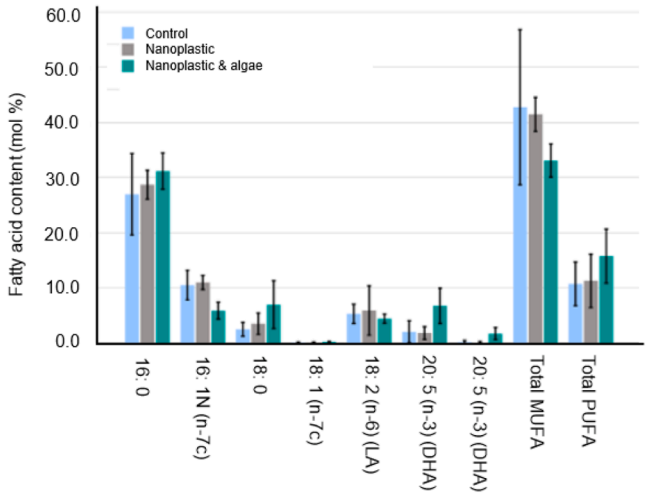

Fig. 2. Fatty acid content (mol %) for key fatty acid markers of embryos at the experiment endpoint. Grouped maternal treatments: Control (blue), Nanoplastic (grey), Nanoplastic & algae (green) are shown since there was no effect of embryo treatment across the experiment. Full maternal and embryo treatment plots for all the identified key fatty acids and lipid measurements can be found in S1 and S2 of the supplementary materials.

incubation water on lipid energy reserves. The impact of plastic particulates on the total lipid reserves of krill has not been previously explored. However, Yoshida et al., (2011), when investigating the effect of maternal diet on krill embryo total lipid and fatty acid composition, found total lipid reserve differed minimally between groups at various embryonic life stages. They also determined total lipid reserve was lowest in the group which also had the greatest hatch success. Together, our data and those of Yoshida et al., (2011) suggest that the quality of lipid is more indicative of healthy embryos rather the quantity of the lipid.

Though the algae only treatment ceased since produced embryos did not develop suitably for the follow-on nanoplastic exposure, we do not believe this to indicative of the treatment conditions since embryos from the nanoplastic plus food treatment developed and the algae used was deemed a suitable food source by Yoshida et al., (2011). Nevertheless, the absence of an algae only endpoint prevents us from drawing full comparisons and herein, our comparisons refer to the nanoplastic only treatment compared to the control, with additional insights provided from the nanoplastic plus food treatment into the influence of a food source on lipid and fatty acid composition. In a previous nanoplastic ecotoxicity study, it has been demonstrated that Antarctic krill is able to feed on nanoplastic agglomerates in the absence of food (Bergami et al 2020), and an experiment without food reflects the natural environmental conditions characterised by the paucity of food resources, such as during the Antarctic winter (Bergami et al 2020).

The conclusion that nanoplastic within incubation water did not affect lipid/fatty acid quality should be interpreted within the experimental context i.e., considering the surface properties of the nanoplastic and its behaviour in Antarctic seawater, as well as the short time <48-hour maternal incubation period. In the present study positively charged polystyrene (PS-NH2) nanoplastic particles were stable in Milli-Q water but agregated in FSW over a 24 hour time period, supassing the nano scale (<1000 nm) after 24 hours at the maternal incubation temperature. Generally positive PS-NH2 particles remain well-dispersed, interacting with biological surfaces and causing high toxicity (Della Torre et al. 2014; Bergami et al. 2017; Pinsino et al. 2017). In the present study, high PDI values (> 0.300) observed in FSW after 24 hours suggest the presence of large aggregates that dominate the light scattering signal, but could also mask the presence of smaller ones. Bergami et al. (2019) also observed agregation of positively charged polystyrene nanoplastic over 24 hours, attributing behavioural differences in part due to physico-chemical properties and low temperatures which can influence the Brownian motion of the particles and consequnetly aggregation states (Jia et al., 2014). The lower absolute ζ-potential values of FSW compared to Milli-Q observed in the present study suggest a screening effect of surface charges due to the higher salt content and presence of proteins, and natural organic matter present in seawater. The same has been observed in other studies (Della Torre et al., 2014). The alteration in surface charge can consequently lead to instability in the dispersal of nanoplastic and fast agglomeration since reduced ζ-potential leads to attractive forces between colloids outweighing the repulsive mechanisms, and the particles can then adhere when they collide (Lin et al., 2010).

The instability of nanoplastic in seawater is an important consideration since particles lose their nano-specific properties, such as their ability to permeate cells, when the size of aggregates exceeds the nano

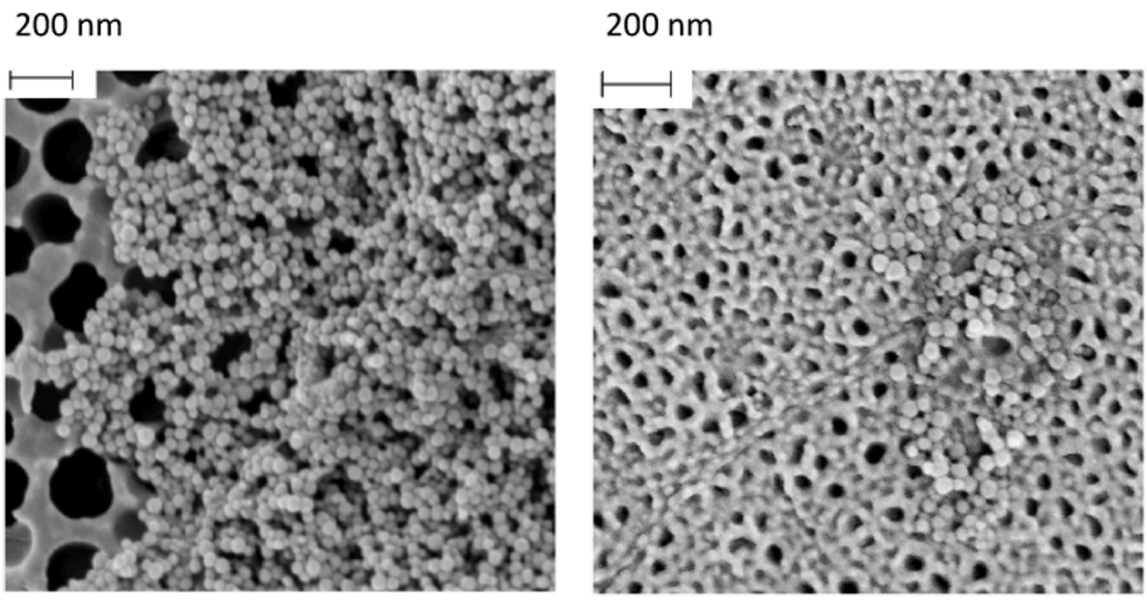

Fig. 3. Scanning electron microscopy example images of digested embryos from (A) maternal control / embryo nanoplastic, and (B) maternal nanoplastic / embryo nanoplastic exposures (Magnification 40.00 kX).

scale, as observed in our experiment. Despite this, we observed nanoplastic within the digested residual of krill embryos both where the mother and the embryos were exposed to nanoplastic, and where the mother was not exposed to nanoplastic but the embryos were directly exposed. Nanoplastic found in digested krill embryo residual is likely due to nanoplastic permeating the outer embryo membrane since a rigours cleaning process of the outer surfaces was adopted. Additionally, using the same methods, Rowlands et al., (2021a) observed no nanoparticles in digested residual of krill embryos exposed to larger 160 nm plastic beads. Similar observations have been observed in other embryo exposure experiments for example, Lee et al. (2019) observed polystyrene nanoplastic internalisation in zebrafish embryos, with 50 nm particles internalised to a much larger extent than 200 and 500 nm particles.

Among the essential PUFAs, linoleic acid, EPA and DHA are considered to be the most important for critical processes such as cell membrane functioning (Persson and Vrede, 2006). DHA is also associated with brain and neurological development during embryogenesis (Tocher et al., 1992) and the EPA/DHA ratio has been determined as a driver of egg quality in marine fish (Sargent et al., 1995). In our experiment, no differences were detected between the nanoplastic only treatment and the control for total PUFAs, EPA, DHA and EPA/DHA ratio. Embryos from the nanoplastic plus algae treatment had significantly higher values for each. Yoshida et al., (2011) determined that total PUFA and DHA/EPA ratio were positively correlated with krill hatch success and levels of 16:1(n-7c) and the ratio SFA/PUFA were negatively correlated with hatching success. We also found embryos from the nanoplastic plus algae treatment had significantly lower 16:1 (n-7c) and saturated fatty acid/PUFA than the control. Collectively, given that incubation water dosed with nanoplastic in the absence of food had no effect compared to the control, our results indicate that the addition of a food source had a positive effect on embryo lipid and fatty acid composition.

Whilst the <48-hour exposure was sufficient time for material of the flagellate to be reflected in fatty acid profiles, the nanoplastic/lipid and fatty acid relationship may be more complex. An intergenerational study exploring how nanoplastic effects fat metabolism, by Cedervall et al., (2012) determined lipid differences in adult fish occurred 22 days after exposure to nanoparticles, concluding that changes in lipid metabolism are slow or that an accumulation of nanoparticles is needed for an effect to be observed. However, with zooplankton species, short-term effects of plastic on lipid have been observed. Cole et al., (2019) noted that exposure to nylon microplastic over 6 days significantly reduced total lipid in preadults of the copepod *Calanus finmarchicus*. In the only other study exploring potential nanoplastic/lipid interactions during the embryonic life stage, Cui et al (2017) observed that *Daphnia galeata*, a fresh water planktonic crustacean, displayed significantly lower hatching rates of embryos exposed to 52 nm polystyrene nanoplastic for five days in the brood chamber. The authors suggested negative effects may be related to nanoplastic induced changes in lipid storage. Whilst short-term exposures to nanoplastic have impacted lipid reserves of other zooplankton species, our observation that nanoplastic did not affect the lipid metabolism of krill should be addressed in the context of the short-term exposure and longer exposures could yield different results.

Extrapolating the potential effects of nanoplastics as a maternal pollutant should be done with caution since lifecycles and breeding strategies differ considerably between species. For example, whilst *D. galeata* reproduce every 8 days, forming eggs in approximately three days (Cui et al, 2017), ovarian development and reproduction for krill is a lengthier process. With a semi-empirical model, Tarling et al., (2007) predicted that the average interval between spawning episodes of female krill in South Georgia was 78 days. Since krill previtellogenesis (the development of the ovary and oocyte), and vitellogenesis (yolk formation via nutrients being deposited in the oocyte) are longer processes occuring over long periods, with oocytes developing over a period of several weeks to 2 months (Ross and Quetin, 1983), embryos may be less susceptible to effects of short-term ecotoxicological exposures. Therefore, since lipid reserves play a key role in the production of krill eggs long before spawning, the effects of long-term nanoplastic exposures should be explored. Further, just prior to the release of an embryo batch (at the time of nanoplastic exposure in the case of our experiment) krill are already beginning to prepare the next set of oocytes for the subsequent embryo batch (Kawaguchi et al., 2007) and therefore analysing the secondary embryo batches post exposure would be a valuable follow up to our experiment.

In addition to lipids, known to be vital for krill development, Amsler and George (1985) determined that krill embryos draw upon protein sources during embryonic development, observing that twice as much protein is used on a weight basis compared to lipid. Future work

exploring the impact of nanoplastic on krill embryo energy reserves should therefore investigate differences in protein composition to complement our exploration of nanoplastic impact on lipid reserves. This may be particularly important since Yoshida (2011) determined that the use of lipid by Antarctic krill during embryonic development is less than observed for other euphausiids (Ju et al., 2006), further supporting protein sources as a main reserve during development. To build further on our results, since embryonic development accounts for <4% of the metabolic requirements of all non-feeding stages, and therefore most energy reserves are passed on to larvae for development through to the first feeding stage (Quetin and Ross, 2003), potential effects of altered energy resources should be explored in the larva stage of krill too.

Following on from maternal treatments, we also aimed to determine whether further direct exposures of embryos to nanoplastic of varying concentrations would alter lipid and fatty acid profiles. We found no significant variation in lipid and fatty acid composition between the control, nanoplastic low dose and nanoplastic high dose embryos and no interaction effects between maternal treatments and embryo treatments. Results align with our previous findings where direct exposure of krill embryos to nanoplastic (160 nm) at the high dose concentration (2.5 μg ml^{-1}) did not impact the probability of reaching the later embryonic development stages (from the development of limbs through to hatching).

In this study we addressed nanoplastic as a single stressor, however, a multitude of anthropogenic climatic stressors (such as ocean warming and ocean acidification) are particularly prevalent in the Southern Ocean (Fabry et al., 2011; Whitehouse et al., 2008). Since biological thresholds can be lower for combined stressors compared to those in singularity, multi-stressor scenarios must also be considered (Rowlands et al., (2021 b). Rowlands et al., (2021 a) determined there was no impact on krill embryo development for the singular nanoplastic stressor. However, when combined with ocean acidification, another prevalent anthropogenic stressor, a statistically significant difference in ability to reach the later development stages was observed when compared to the control of ambient seawater. With another Antarctic species, Manno et al., (2022) determined that co-exposure to both nanoplastic and ocean acidification increased the mortality rate of the pteropod Limacina retroversa further highlighting that plastic pollution needs to be addressed in the context of global climate change.

Antarctic krill supports various ecosystem services e.g., as a food source for vertebrate predators (Trathan and Hill, 2016), promotes carbon sequestration into the deep ocean (Manno et al., 2020) and is a dominant stock for the fishing industry (Nicol et al., 2012). However, ocean warming, sea ice decline, and other environmental stressors act simultaneously to modify the abundance, distribution, and life cycle of krill (Flores et al., 2012). Our study is the first to scope intergeneration effects of nanoplastic on the lipid and fatty acid reserves of krill embryos. We highlight that krill embryos appear resilient to polystyrene 50 nm nanoplastic under short-term exposure conditions. Results should be further extrapolated with caution since the toxicity of nanoplastic is known to differ based on surface functionality, particle size and polymer type (Blasco, J., Corsi, 2021). Future studies should consider the impact of nanoplastic on other critical energy reserves such as proteins and explore the potential effect of long-term exposures on both lipid and protein composition/reserves. Finally, investigating the impact of nanoplastic multi-stressor scenarios on the energetic resources of krill is another important future step.

Author contributions

ER and CM conceptualized the study. ER conducted the experimental design and field experiments with the support of CM. ER carried out the original drafting of the manuscript. CH conducted nanoplastic internalisation analyses. SB conducted lipid extractions and GCMS analysis. ER, CM, SB and GW were involved with the data interpretation. ER conducted the data analysis. TG, MC, CL, VP, and ST provided a general overview. All authors contributed to the redrafting, reviewing, and editing of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106591.

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